

Molecular identification and cultural characteristics of *Penicillium citrinum* associated with marine dried fish of *Stolephorus commersonnii* (Lacépède, 1803)

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Abstract

Stolephorus commersonnii is a common marine fish popular in Bangladesh's coastal regions as dried fish. The goal of this study was to identify the fungus by molecular techniques, including its morphological characteristics and cultural factors associated with dried fish. Mycelium, hyphae, and distinctive brush-like branching conidiophores with a long chain of conidial morphology were used to identify *Penicillium citrinum*, which was isolated from *S. commersonnii*. Additionally, the internal transcribed spacer region (ITS) of *P. citrinum* was analyzed. A 100% sequence match with the *P. citrinum* species complex was revealed by the polymerase chain reaction (PCR) results for *P. citrinum* isolate JUF0137 and its *GenBank* accession number PZ251380.1. Potato dextrose (PDA) medium (75 mm) and yeast extract agar medium (31 mm) exhibited the highest and lowest mycelial growth rates, respectively. According to the analysis of fungal growth characteristics, *P. citrinum*'s mycelial development was most suitable at 30°C and pH 7. *P. citrinum* from *S. commersonnii* has never been reported in Bangladesh, as far as it is concerned.

Key words: Anchovies, Dry fish fungus, Molecular identification, Mycelial growth, *Penicillium citrinum*.

INTRODUCTION

Stolephorus commersonnii (Lacépède, 1803), which is also referred to as anchovies, is a member of the Clupeiformes order and the Clupeoidei suborder, which includes four families (Kamukuru *et al.*, 2020). Most small fish species found in the world's coastal ecosystems and fisheries belong to two of these families: anchovies (Engraulidae) and sardines or herrings (Clupeidae). Anchovies have been shown to have various biological traits, including short plankton-based food chains, rapid growth, short lifespans, relatively low fecundities, and year-round spawning in some species, making them particularly sensitive to environmental change (Checkley *et al.*, 2017). According to Guénette *et al.* (2014), anchovies are essential to the ecosystem because they repackage energy used by higher trophic levels. Nonetheless, these small marine fish species are thought to be

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resilient and able to withstand high levels of predation and fishing pressure due to their rapid turnover rates.

Chittagong, Bagerhat's Dublar Chor, Moheshkhali, Sonadia, and St. Martin Island in Cox's Bazar, and Patuakhali's Kuakata are the most important places for dried fish production (Sultana *et al.* 2020). Dried fish and fish items are made from a variety of marine fish species as well as freshwater fishes. Bangladeshi dry fish has enormous commercial potential since it contains high-quality proteins, necessary vitamins, and important polyunsaturated fatty acids. Nowadays, dry fish items of *S. commersonnii* are gaining popularity among Bangladeshis due to their ready-to-eat nature, composition, and flavor (Ara *et al.*, 2020a).

The easiest, most traditional, least expensive, and oldest way to preserve fish is sun drying, which is especially popular in rural areas. (Masud *et al.*, 2023). In Bangladesh, people are far more aware of health concerns, and the quality of traditionally processed dried fish and fish products is considered a public health concern. Taking advantage of this nature of the Bangladeshi people, the fish processing industry is now prioritizing value addition over quality control in order to increase profits. Microbial contamination may constitute a concern to public health in addition to nutritional degradation. According to Thiyagarajan & Jamal (2021), research on the mycological investigation of dried fish gathered from open markets, six kinds of fungi were found to be common, including *A. parasiticus*, *A. niger*, *A. fumigatus*, *Euratum* sp., *Penicillium* sp., and *Cladosporium* sp.

Numerous fungus species including *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp., and *Fusarium* sp that seriously deteriorate dried fish have been identified (Chanda *et al.*, 2020; Ara *et al.*, 2020b; Akter *et al.*, 2023). If appropriate safety measures are not taken, dried fish can be attacked by fungi during the drying and storage stages, which can lead to spoilage of the fish and pose a health risk. *Aspergillus* and *Penicillium* species are among the most prevalent types of fungi detected in dry fish. They may produce a variety of mycotoxins, including liver-damaging and carcinogenic aflatoxins. In China, fungal diversity and mycotoxin contamination in dried fish products were investigated. *Aspergillus* sp. (63.9%), *Penicillium* sp. (70.7%), and *Fusarium* sp. were the three most common genera in dried fish out of 25 strains from 12 genera. Contamination with *A. flavus* varied between 1.10×10^3 and 2.40×10^4 cfu/g (Deng *et al.*, 2021). An evaluation of the fungal contamination of dried fish sold in open marketplaces revealed that the majority of dried fish had multiple fungal contaminations. *Penicillium* spp., *Aspergillus* spp., and *Fusarium* spp. were identified as significant contaminants (Chowdhury *et al.*, 2025). Nevertheless, there is a lack of comprehensive data regarding the fungal contamination of *Stolephorus commersonnii* marine dried fish in Bangladesh. Therefore, the current investigation's objective was to identify any potential fungal contamination associated with *S. commersonnii*.

MATERIALS AND METHODS

Collection and preservation of samples: Fresh and dried *Stolephorus commersonnii* samples (Fig. 1A, B) were collected from Kuakata, Patuakhali, a coastal fish landing and

drying center in Bangladesh. The fresh fish were thoroughly washed with tap water before being sun-dried for 15 days in natural conditions. After drying, the samples were placed in a sterile glass jar and sent to the Mycology and Plant Pathology Laboratory, Department of Botany at JU's University, for fungal inoculum isolation and identification.

Isolation and identification: Each of the minuscule, 0.5 cm-long contaminated sections from both fungus-infested and uninfested dried fish segments were isolated. These microscopic bits were placed in a 90mm Petri dish filled with potato dextrose agar (PDA) medium without being sterilized after being repeatedly rinsed with distilled water. The fungal colonies were subsequently monitored for ten days at room temperature under a 12-hour light/12-hour dark cycle. After numerous transfers of the hyphal tip, a pure culture was created. Standard procedure were used to identify the pure culture of the isolated fungus under a microscope (Ahmmed *et al.*, 2022).

Molecular characterization: Molecular identification and characterization of the studied fungal species was carried out by the commercial services of Invent Technology, Dhaka, Bangladesh. To extract genomic DNA from fungus, the Maxwell Cell Kit (AS1030, Promega, USA) was used. 20 ng of genomic DNA was used as the PCR template in a 25- μ l reaction mixture with a LA Taq (TAKARA BIO INC., Japan). After 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for five minutes each, with a ten-minute extension at 72°C, Taq polymerase was activated at 94°C for one minute. The primers used for the PCR reaction were ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAA AGTCG TAACAAGG-3') (Alam & Rahman, 2020).

The amplified PCR products were electrophoresed on a 1.5% agarose gel in 1 $\times$ TAE buffer for one hour at 100V using a 1kb DNA ladder as a size marker and stained while being swirled in an EtBr solution (0.5% μ g/mL). The Stained gels were then placed in a UV transilluminator (Kodak Image Station 4000R; Molecular Imaging Equipment, Carestream Health Inc., 150 Verona Street, Rochester, NY 14608) for observation and photography. The Maxwell® 16 DNA Purification Kits (Promega, USA) were used to purify the amplified products. First, the purified PCR products were subjected to bi-directional sequencing by BASE Laboratories Sdn Bhd (Malaysia).

DNA sequences were verified using MEGA 11 and Bio Edit. The ITS sequences were used in a BLAST search to find the closest matched taxa. Multiple sequence alignments were performed using MEGA6. The data was converted from Fasta to MEGA format using Clustal W. The evolutionary models were defined using the Akaike Information Criterion (AIC). The Tamura-3 parameter served as the model for the analysis. Using 1000 bootstrap repetitions and max-trees set to 1000, the robustness of the branches was evaluated. Analyses of neighbor-joining were performed. The number of replicates was calculated using the halting criterion. Bootstrap values greater than 60% were considered acceptable, according to Tamura *et al.* (2013).

Culture media, temperature, and pH's consequences for mycelial growth: Five distinct culture media, including potato sucrose agar (PSA), potato dextrose agar (PDA), glucose

peptone (GLP), yeast extract agar (YEA), and Hopkins (HOP) medium, have been used to assess the mycelial growth characteristics of the isolated fungi, according to Alam *et al.* (2023). To determine the ideal temperature for mycelial growth, inoculated PDA plates were incubated at 15, 20, 25, 30, and 35 °C. Besides a range of pH levels, including 5.0, 6.0, 7.0, 8.0, and 9.0, was employed to find out the effect of pH on the pathogen's growth using the PDA medium. Before autoclaving, the medium was pH-adjusted with 1 N NaOH or HCl and then incubated at 30°C for ten days. The average colony diameter was calculated by measuring the radial mycelial development of each Petri plate in three opposing directions. Each experiment was performed using the method described by Sikder *et al.* (2019).

Data analysis: Standard statistical analysis tools like Microsoft Excel, MEGA 11.0, and BLAST were used to examine the data produced during the research work. The data was examined using Duncan's post-hoc analysis.

RESULTS AND DISCUSSION

Identification based on morphology: *Penicillium* is distinguished by rapidly expanding, velvety colonies that frequently have a distinctive reverse side color and seem blue-green or white (Fig. 1). Hyaline hyphae and characteristic brush-like branching conidiophores (penicillin) bearing a lengthy chain of conidia are its microscopic characteristics (Fig. 1). The conidiophores with solitary phialides of species in section *Torulomyces* and the darkly pigmented stipes that once distinguished the genus *Thysanophora*, which show secondary growth through the proliferation of an apical penicillus, were most notably included. It now excludes the acrose phialides and typically symmetrically branched conidiophores of the species (Visagie *et al.*, 2014).

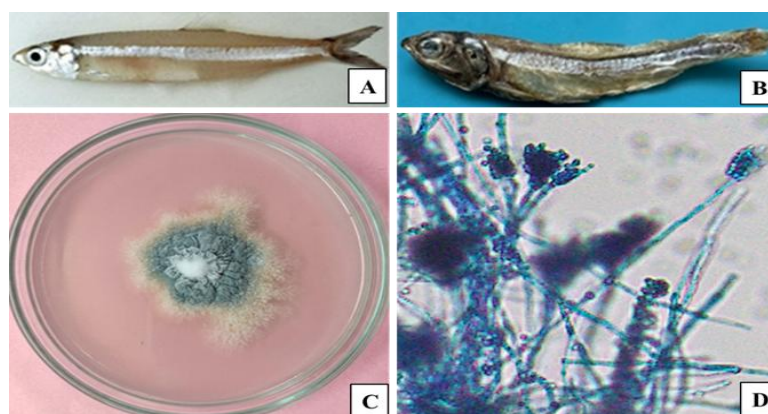


Fig.1. Morphological features of *Penicillium citrinum*, isolated from dried *Stolephorus commersonnii*. A, fresh *S. commersonnii* fish; B, dried *S. commersonnii* fish; C, colony structure on PDA medium; D, hyphae, conidiophore, phialides and conidia (40x).

Molecular Characterization of *Penicillium citrinum*: The 588 bp ITS region was amplified using ITS1 and ITS4 primers before being sequenced (Fig. 2). Recent molecular

phylogenetic studies have demonstrated that the ITS region of genomic DNA is very useful for recognizing fungi at lower taxonomic groupings. The internal transcribed spacer of rDNA is thought to vary between species and even between strains (Alam *et al.*, 2010a).

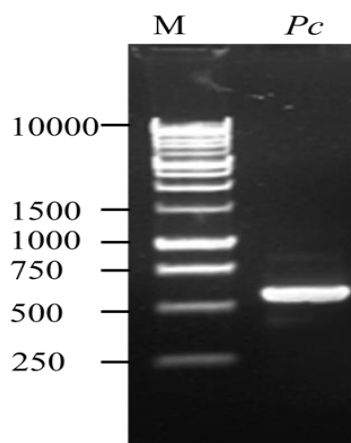


Fig. 2. Amplification profiles of the tested fungus's rDNA-internal transcribed spacer region. M, molecular size marker (1kb DNA ladder); Pc, *Penicillium citrinum*.

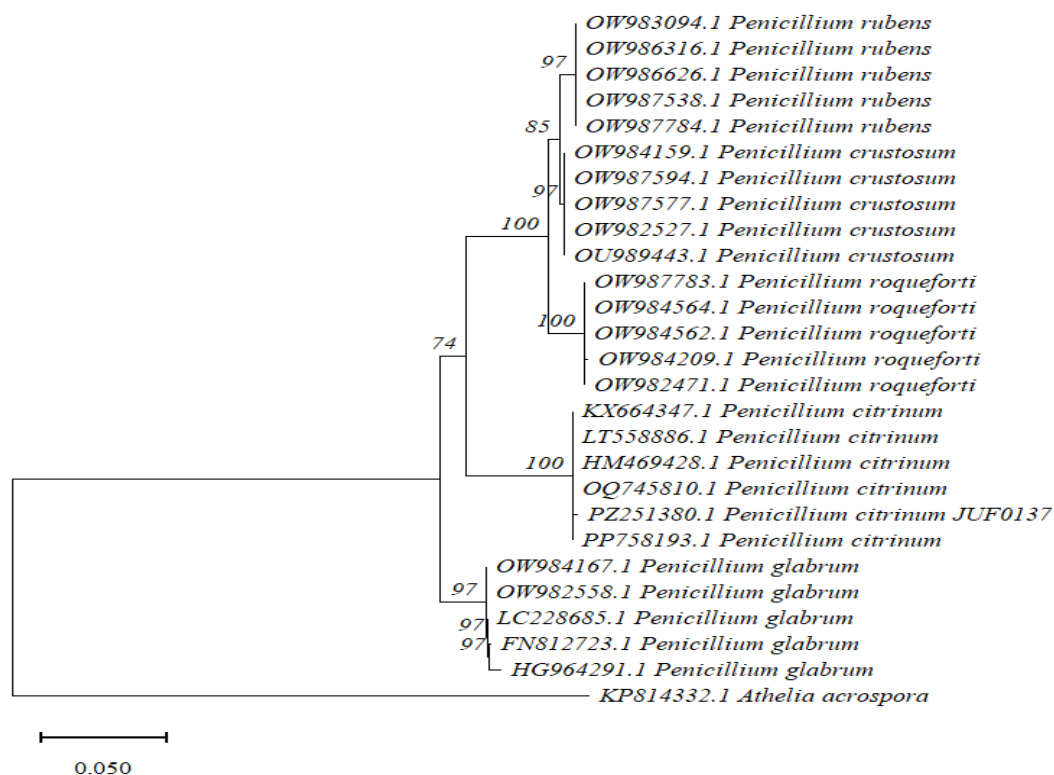


Fig. 3. *Penicillium citrinum* neighbor-joining tree based on bootstrap value analysis of the ITS sequence

The BLAST search study revealed that the examined organism, *P. citrinum*, is identified as PZ251380.1 (JUF0137) and has 99% identity with *P. citrinum* (PP758193.1). The NCBI database provided twenty-seven fungal species, and the nucleotide sequences of the ITS components were used to generate a phylogenetic tree. Using neighbor-joining methods, the phylogenetic tree revealed five distinct clades (Fig. 3). According to the findings, each distinct *Penicillium* species is associated with a specific cluster. The available information clearly indicates that the fungus is *P. citrinum*. Reciprocal homologies ranging from 100% were found in the ITS region sequences. For phylogenetic association analyses with the identified *P. citrinum* strain (JUF0137), the sequencing data of the designated NCBI GenBank strain (KP814332.1 *Athelia acrospora*) were applied as an outgroup.

According to Alam *et al.* (2010b), ITS sequences are genetically stable within species but vary between species within a genus. DNA sequences are necessary for reliable identification of the species *Penicillium*, according to Visagie *et al.* (2014). The genus currently lacks a complete, validated reference database. To improve the list's taxonomic value, we additionally provide the MycoBank number of each accepted species, live ex-type strains, and GenBank accession numbers for ITS sequences. This generates a confirmed set of sequences for each species in the genus (Alam *et al.*, 2010c).

Effect of culture media, temperature, and pH on *Penicillium citrinum*: The results of five different culture media for the mycelial growth have been presented in Fig. 4. *P. citrinum* had the largest mycelial growth (75mm) on PDA medium, followed by PSA medium (57mm), GLP medium (55mm), HOP medium (43mm), and YEA medium (31mm). The current *P. citrinum* results are supported by Kar *et al.* (2025), who found that potato dextrose agar culture medium was the best solid culture medium for mycelial growth and development of *Aspergillus elegans*. According to the findings, PDA medium produced the optimum mycelial growth pattern in vitro. Similar results were also reported by Akter *et al.* (2023) in *A. ochraceopetaliformis*, which was isolated from small indigenous dry fish of *Setipinna phasa*.

The current study examined whether temperature affected the mycelial growth of *P. citrinum* on PDA media. Fig. 5 shows the results of incubating the medium at five different temperatures (15°C, 20°C, 25°C, 30°C, and 35°C). According to the experimental findings, the maximum mycelial growth of *P. citrinum* was recorded at 30°C, then 25°C. Our experiment indicated that *P. citrinum* grew best at 30°C, that is similar to the findings of Babu *et al.* (2015) where *Penicillium menonorum* had shown the strongest mycelium growth and sporulation at 30°C. The data also demonstrated that temperature has no effect on the mycelial growth of *P. menonorum*, and that the optimal temperature can be used to minimize the growth of the tested fungus while maintaining quality.

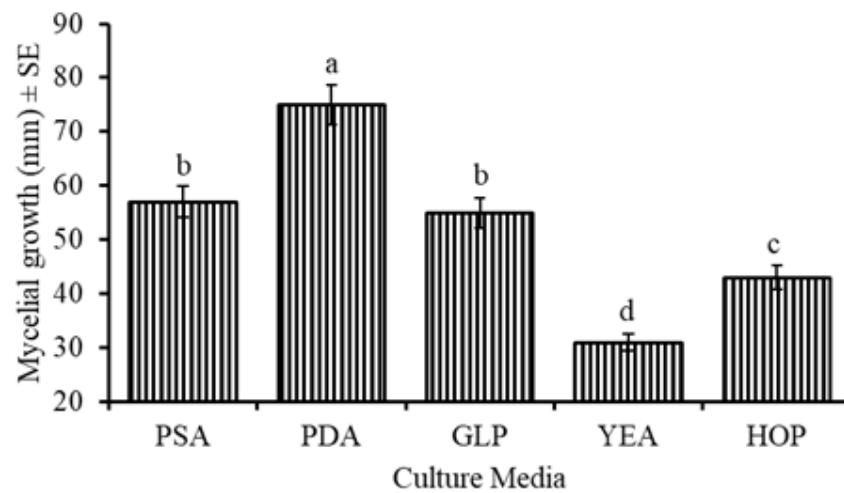


Fig. 4. The optimum growth of *P. citrinum* in various culture media at 7 dpi. The bars in the figure show the means $\pm$ standard errors ($n = 3$). Significant differences between treatments ($p < 0.05$) are shown by different letters

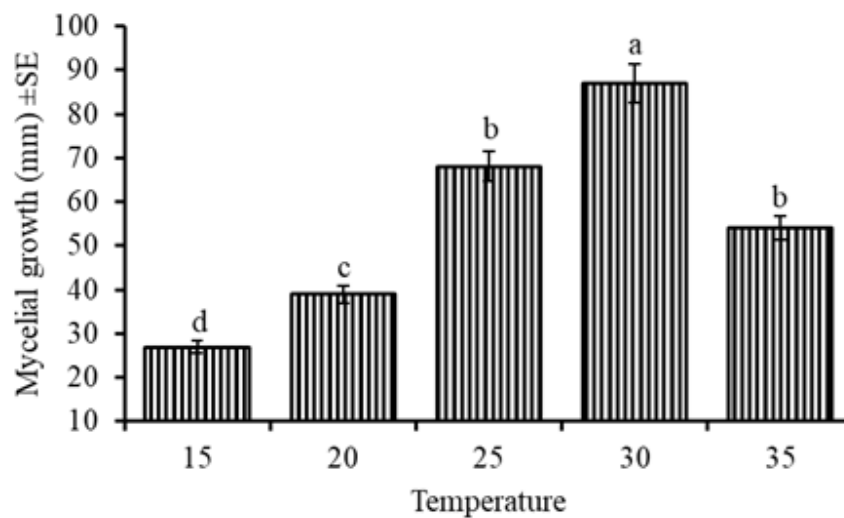


Fig. 5. The optimum growth of *P. citrinum* in various temperatures at 7 dpi. The bars in the figure show the means $\pm$ standard errors ($n = 3$). Significant differences between treatments ($p < 0.05$) are shown by different letters

A crucial factor in comprehending the fungal ecology is pH. However, the experimental plates were incubated at five different pH levels: 5, 6, 7, 8, and 9. *P. citrinum* showed maximum mycelial growth (75mm) at pH 7, followed by pH 6 (46mm) and pH 8 (39mm), and minimum mycelial growth (21mm) at pH 9 (Fig. 6). Visagie *et al.* (2015) reported that the maximum growth of *Penicillium menonorum* at pH 7.0, which showed very similar from our experimental findings. Sultana *et al.* (2023) noticed the maximum mycelial

growth of mold fungi when the pH of the media was between 6.5 and 7.5. As a result, the findings revealed that *P. citrinum* prefers neutral pH conditions.

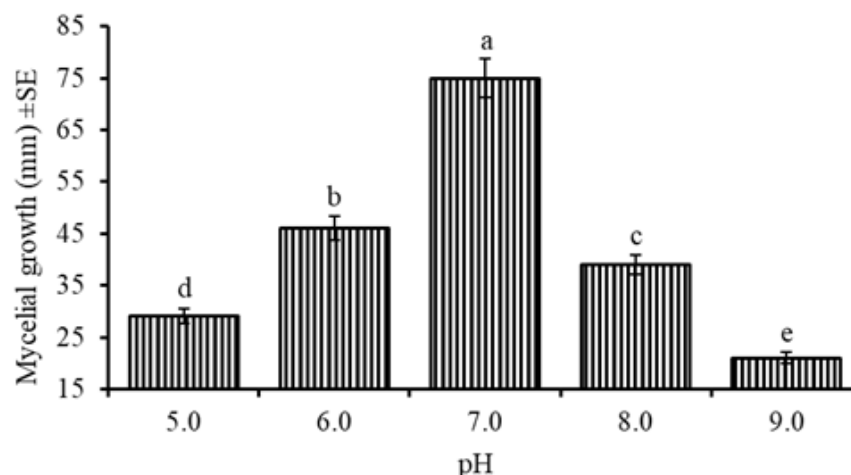


Fig. 6. The optimum growth of *P. citrinum* in various pH at 7 dpi. The bars in the figure show the means $\pm$ standard errors ($n = 3$). Significant differences between treatments ($p < 0.05$) are shown by different letters

This research concluded that most of the dried fish samples were contaminated with fungi. *Penicillium* is a fluctuating genus that is found all over the world. Its species are major organic material decomposers and can cause harmful rots in food processing. The current study is a groundbreaking inquiry into the fungi that are linked to dried small native marine fish species in Bangladesh.

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